RECEIVED
CENTRAL FAX CENTER
APR 0 4 2007

Attorney Docket No. 5051.338CTDV

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Conkling et al.
Serial No.: 10/748,789
Filed: December 30, 2003
For: *Regulation of quinolate phosphoribosyl transferase expression*

Group Art Unit: 1638
Examiner: R. Kallis
Confirmation No. 9424

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Declaration of Dr. Takashi Hashimoto
Pursuant to 37 C.F.R. § 1.132

I, Takashi Hashimoto, do hereby declare and say as follows:

1. I have a Ph.D. degree from Kyoto University in Kyoto, Japan. I am currently a Professor in the Graduate School of Biological Sciences at the Nara Institute of Science and Technology (NAIST), located at 8916-5 Takayama, Ikoma 630-0101 Nara, Japan. I have been in this position for 7 years and have been employed by NAIST for 13 years.

I have been the Principal Researcher of a research project entitled "Controlling Total and Individual Alkaloid Levels in *Nicotiana* Plants," ("RESEARCH") which is supported by 22nd Century Limited, LLC ("22nd Century"). To the best of my knowledge, 22nd Century is the exclusive licensee of U.S. Application No. 10/748,789.

I have conducted research in the field of alkaloid biosynthesis in plants, including nicotinic alkaloid biosynthesis in tobacco plants, for 15 years and have authored or co-authored about 13 publications on this topic.

2. The following studies were conducted under my direction and supervision within the RESEARCH:

a. *Increasing nicotine content by expression of QPT under control of the TobRD2 promoter*

The QPT ORF (Figure 4) was transferred from a pDONR221-QPT vector to a GATEWAY® binary vector pTobRD2-DEST (Figure 1C) by an LR reaction. The gene expression vector was referred to as pTobRD2-QPTox (Figure 1D). The plasmid pTobRD2-QPTox was transformed into *Agrobacterium tumefaciens* strain EHA105, which was used to transform wild-type K326 leaves. Transgenic T0 shoots were regenerated and transferred to rooting medium. Several rooted transgenic plants were transferred to soil.

Transgenic hairy roots and/or tobacco leaves were collected and freeze dried. Two ml of 0.1 N sulfuric acid was added to 19 mg of the freeze-dried sample. This suspension was sonicated for 15 minutes and filtered. Ammonium hydroxide (0.1 ml, 28% NH_3; Wako) was added to 1 ml of the filtrate and the mixture was centrifuged for 10 minutes at 15,000 rpm. A sample of the supernatant (1 ml) was loaded onto an Extrelut-1 column (Merck®) and allowed to sit for 5 minutes. Alkaloids were eluted with 6 ml of chloroform. The eluted chloroform fraction was then dried under reduced pressure at 37°C with an evaporator (Taitec Concentrator TC-8). The dried sample was dissolved in 50 µl of ethanol solution containing 0.1% dodecane. A gas chromatography apparatus (GC-14B, Shimadzu®) equipped with a capillary column (Rtx-5Amine column, Restec) and an FID detector was used to analyze the samples. The column temperature was maintained at 100°C for 10 min, elevated to 150°C at 25°C/min, held at 15 °C for 1 min, elevated to 170°C at 1°C/min, held at 170°C for 2 min, elevated to 300°C at 30°C/min, and then held at 300°C for 10 min. Injection and detector temperature was 300°C. A 1 µl sample of the purified alkaloid preparation was injected and alkaloid contents were measured by the internal standard method.

Alkaloids were extracted from the transgenic tobacco leaves and analyzed, as described herein. The nicotine content in leaves of plants sampled 36 days after transfer to soil was analyzed. Several transgenic lines showed greater nicotine accumulation than the control lines, in which wild-type K326 plants were transformed with a TobRD2-GUS cassette. (Figure 3).

b. *Increasing nicotine content by expression of QPT under control of the A622 promoter*

The QPT ORF was amplified using the pBJY6 vector (supplied from Dr. Kenzo Nakamura, Nagoya University, Japan) as the template and the gene-specific primers shown below. A GATEWAY® entry clone pENTR-QPT was created by a TOPO® cloning reaction.

QPT gene-specific primers:
QPT-F 5' CACCATGTTTAGAGCTATTCC
QPT-R 5' TCATGCTCGTTTTGTACGCC

The QPT ORF was transferred from the pENTR-QPT vector to the GATEWAY® binary vector pA622pro-DEST (Figure 1A) by an LR reaction. The gene expression vector was referred to as pA622pro-QPTox (Figure 1B). The plasmid pA622pro-QPTox was transformed into *Agrobacterium tumefaciens* strain EHA105, which was used to transform wild-type K326 leaves. Transgenic T0 shoots were regenerated and transferred to rooting medium. Several rooted transgenic plants were transferred to soil.

Alkaloids were extracted from the transgenic tobacco leaves and analyzed per above. The nicotine content in leaves of plants sampled 36 days after transfer to soil was analyzed. Several transgenic lines showed greater nicotine accumulation than the control lines, in which wild-type K326 plants were transformed with an A622-GUS cassette (Figure 2).

Prior work by others has shown that leaf nicotine levels and root QPT activity are highly correlated (Saunders and Bush, Plant Physiol 64:236,1979). Based on my previous experience with nicotine biosynthesis genes, when the expression level of a gene is increased, the enzyme

activity of the gene product is also increased. Therefore, my expectation is that the expression level of the QPT gene and the enzyme activity of QPTase are increased in the transgenic plants described here.

3. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Takashi Hashimoto
Dr. Takashi Hashimoto

March 23, 2007
Date